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Partial purification and chemical characterization of a glycoprotein (putative hydrocolloid) emulsifier produced by a marine bacterium *Antarctobacter*

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Abstract During screening for novel emulsifiers and surfactants, a marine alphaproteobacterium, *Antarctobacter* sp. TG22, was isolated and selected for its production of an extracellular emulsifying agent, AE22. This emulsifier was produced optimally in a low-nutrient seawater medium supplemented with glucose and was extractable by cold ethanol precipitation of the high-molecular-weight fraction (>100 kDa). Production of AE22 commenced towards the late exponential phase of growth, with maximum emulsifying activity detected after approximately 4 days of the cells entering the death phase. Chemical, chromatographic and nuclear magnetic resonance spectroscopic analysis confirmed AE22 to be a high-molecular-weight (>2,000 kDa) glycoprotein with high uronic acids content, thus denoting an apparent polyanionic structure. Functional characterization showed this polymer to compare well to xanthan gum and gum arabic as an emulsion-stabilizing agent for a range of different food oils. However, AE22 exhibited better stabilizing than emulsifying properties, which could be conferred by its viscosifying effect in solution or from certain chemical groups found on the polysaccharide or protein moieties of the polymer. This new high-molecular-weight glycoprotein exhibits interesting functional qualities that are comparable to other biopolymers of this type and shows particular promise as an emulsion-stabilizing agent in biotechnological applications.

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Introduction

Surface-active agents are a group of amphipathic chemical compounds (i.e. they have both hydrophobic and hydrophilic domains) distinguished for their ability to interface between water-soluble and oil components (Banat et al. 2000; Desai and Banat 1997; Singh and Cameotra 2004). They form an indispensable component in almost every sector of modern industry, with total global production estimates exceeding 3 million tonnes per year (Banat et al. 2000). A major limitation in their use derives from the fact that most of these compounds are synthetically manufactured from organo-chemical synthesis from hydrocarbons, raising concerns over their environmental impact and potential health effects (Desai and Banat 1997). Naturally derived surface-active agents (i.e. biosurfactants and bioemulsifiers), however, have gained increasing interest in recent years because of their associated lower levels of toxicity, higher degradability, and consumer demand for natural alternatives (Banat et al. 2000; Weiner 1997).

Naturally derived high-molecular-weight surface-active agents or amphipathic biopolymers are an important class of chemical compounds. They have a number of advantages

over their lower-molecular-weight counterparts, examples of which are: (1) a larger surface area to which a greater number of reactive groups can be expressed (Rosenberg and Ron 1997), (2) good texturizing and stabilizing properties that help to slow down phase separation (Klekner and Kosaric 1993; Shepherd et al. 1995), (3) tensile strength and resistance to shear (Garti 1999; Hasenhuettle and Hartel 1997) and (4) there is evidence suggesting health-enhancing or nutritional qualities (Hosono et al. 1997; Nakajima et al. 1992; Ruijssenaars et al. 2000). Gum arabic, for example, is one of the most important of these biopolymers, used extensively as an essential component in citrus beverages. It is defined as a hydrocolloid emulsifier for its ability to both emulsify and stabilize oil-in-water emulsions. However, despite this dual functionality, gum arabic has a 'low yield value' (Sanderson 1990). That is, relatively high concentrations of the polymer are needed (up to 20% w/v) to achieve optimal functionality (Dickinson et al. 1988; Randall et al. 1988).

The high-volume demand for a product or process ingredient can impact heavily on manufacturing and end-product costs, as well as the source of its supply. This has been evident in recent years, notably gum arabic and locust bean gum, which have been the subject to a number of price fluctuations mainly because of a shortfall in their supply from low crop yields and increasing energy and transport costs. In this respect, there is increasing interest directed towards alternative and more reliable ingredients. One method that has been explored is to design new techniques for over-producing and selective-tailoring of these polymers, such as the use of recombinant deoxyribonucleic acid (DNA) technology to express them in vitro using alternative host systems (Xu et al. 2005). Such methods are promising, yet they are still reliant on the genetic and biochemical limitations of the recombinant producing strain. Traditional methods of screening from natural environments, particularly from relatively unexplored niches, still continues to be a reliable tool for isolating and increasing our inventory of new biopolymers that may reveal potentially novel chemical and physical properties (Banat et al. 2000; Weiner 1997).

In this report, we describe the partial purification and chemical characterization of an apparently novel high-molecular-weight glycoprotein emulsion stabilizer produced by a species of *Antarctobacter* isolated from a marine water sample. Compared to commercial hydrocolloids, this new polymer exhibited an enhanced capacity to produce stable oil-in-water emulsions with a range of different food-grade oils. Properties associated with the chemical characteristics and viscosifying effect of this polymer are discussed in view of its interesting emulsion-stabilizing effects.

Materials and methods

Isolation, growth, identification and screening

Bacteria were isolated by spreading 100 μl of tenfold serial dilutions of seawater on solid synthetic seawater medium (Passeri et al. 1992) supplemented with NH_4NO_3 and *n*-hexadecane supplied via the vapour phase as the sole carbon source. Colonies were grown in the dark for 3 weeks at 28°C. Isolates displaying distinct colony morphologies were streaked onto ZM/10 agar (Green et al. 2004) and stored at −80°C with 30% glycerol. Isolate TG22 was selected for its production of high emulsifying activity and identified by DNA sequencing of its 16S ribosomal DNA (rDNA) genes as described previously (Green et al. 2004). Strain TG22 was deposited in the National Collection of Industrial and Marine Bacteria (NCIMB) as strain number NCIMB 41245.

Testing for production of surfactants and/or emulsifiers by isolate TG22 was performed during growth of the bacterium in ZM/10 and ZM/1 liquid medium amended with *n*-hexadecane, glucose or no added carbon source. Hexadecane was added at 3% (v/v) and glucose at 1% (w/v). Samples were taken periodically for emulsification assay and tensiometry (see below). Both whole-broth cultures and cell-free supernatants (13,000×*g*; 10 min) were assayed. Viable cell counts were measured by plating out appropriate dilutions of culture broth onto ZM/10 agar plates.

Production and extraction of emulsifiers

Strain TG22 was grown in 2-l Erlenmeyer flasks containing 770 ml of ZM/10 medium amended with glucose (1% w/v) and incubated (28°C; 150 rpm) to the stationary phase of growth (120–145 h) when maximum emulsification activity was detected in the cell-free culture liquid. The cultures were then pooled together, and the emulsifying fraction was extracted by removing the cells using cross-flow filtration (0.2 μm; Schleicher and Schuell) and then passing the cell-free permeate through a 100-kDa membrane cassette (Schleicher and Schuell). The 100-kDa retentate was dialysed against 10 l of distilled water, and the emulsifying fraction was precipitated with KCl (7.5% w/v) and 3 vol of cold 99% ethanol. The precipitated material was recovered by centrifugation, dialysed against 10 l of distilled water and then lyophilized. The resultant dried material was used in all subsequent chemical and physical characterization experiments.

Emulsification assays

A modified version of the method described by Cooper and Goldenberg (1987) was used to measure emulsifier produc-

tion. Samples (0.5 ml) to be tested were introduced into acid-washed (0.1 N HCl) screw-cap glass tubes (100× 13 mm) and then overlaid with an equal volume of *n*-hexadecane. The tubes were manually shaken (15 s) and vortexed (15 s) vigorously to homogeneity, allowed to stand for 10 min, shaken again as before and then allowed to stand for 24 h at 21°C. The height of the emulsion layer (EI_{24}) was then measured and expressed as a percentage of the total original height of oil and water in the tube.

The ability of the extracted emulsifier AE22 to form oil-in-water emulsions was determined using a modified version of the method described by Cirigliano and Carman (1984). For this, aqueous solutions of the emulsifier (0.2% w/v) were mixed with the test oil (20% v/v) in the same way as described above. The mixtures were allowed to stand for 10 min before taking turbidity measurements of the bottom layer using a spectrophotometer at 540 nm. Triplicate readings were taken every 10 min for up to 60 min, and the log of these plotted against time. The slope of the curves generated were calculated and expressed as the decay constant (K_d), which is a term that describes the stability of the emulsions formed, as previously described (Cirigliano and Carman 1985). The emulsifying activity (A_{540}) was recorded after allowing the emulsions to stand for 24 h. All K_d and A_{540} values were expressed as the average of triplicate experiments. The commercial emulsifiers xanthan gum and gum arabic were used as controls. Activities were compared under neutral (0.1 M phosphate-buffered saline, pH 7.5) and acidic (0.1 M sodium acetate buffer, pH 3.5) conditions.

Tensiometry

Surface tension measurements were performed using a Nima DST-9005 tensiometer by the Wilhelmy plate method to initially screen for surfactant production. The du Noüy ring method was used to measure surfactant production by strain TG22 during growth in liquid medium and to determine if its produced emulsifier, AE22, could reduce the surface tension of water across a range of different concentrations.

Chemical analysis

For determination of the monosaccharide composition, triplicate volumes (10 μl) of 1% solutions of the AE22 extract were dissolved in 500 μl of 2 M trifluoroacetic acid and hydrolysed for 4 h at 100°C. The samples were subsequently prepared for high-performance anion exchange chromatography analysis using a Dionex Carbopac PA-20 column, and the monosaccharides were quantified using external standards. The total carbohydrate content was calculated from the individual amounts of monosaccharides.

For determination of amino acid composition, acid hydrolysis and derivatization was performed on triplicate volumes (100 μl) of the AE22 extract (1% w/v). Samples were hydrolysed in 1 ml of acid (6 N HCl; 112°C for 22 h) and then prepared for analysis using a Waters Alliance high-performance liquid chromatography (HPLC) system equipped with a Zorbax XDB C18 reverse phase column. Detection was carried out using a fluorescence detector (λ= 250 nm; λ=395 nm), and quantification was performed using external standardization with amino acid standard mixtures. The total protein content was calculated from the individual amounts of amino acids.

Lipid analysis was performed on three equal amounts of AE22 and analysed by gas chromatography (GC), as described previously (Cook et al. 2000).

For proton nuclear magnetic resonance (NMR) analysis, approximately 5 mg of the AE22 extract was lyophilized twice from D_2O 99.9% (Apollo Scientific, Stockport, UK), then taken up in 0.7 ml of the same solvent with the addition of 1 μl of a 2% solution of acetone in D_2O as an internal reference. Proton NMR spectra were recorded at 60°C using a Varian Inova 500 MHz spectrometer.

Deproteinization of the AE22 polymer was attempted with the use of the enzyme, alcalase. For this, approximately 1 mg/ml solution of the polymer in water was treated with 0.025 U of alcalase (Novozymes, Denmark) for 1 h at 60°C and then heated to 100°C for 10 min to inactivate the enzyme. Control experiments excluding the addition of the polymer or enzyme were included. The samples were then stored at −20°C before chromatographic analysis.

Chromatographic analysis

To determine the purity of the AE22 extract and obtain an estimate on the molecular mass (M_r) of the emulsifying/stabilizing component, high-performance size-exclusion chromatography was performed with an Agilent 1100 chromatograph equipped with a refractometer and diode-array UV detector. A PL gel filtration chromatography column (Polymer Laboratories; 7.5×300 mm) was used at 30°C. The mobile phase used was either 0.1 M $NaNO_3$ (pH 8) or filtered seawater (FSW), as specified, and the flow rate 0.6 ml/min. Dextran standards of M_r range 1,270–1,400,000 Da (Sigma) were used to calibrate the column for molecular weight estimation. Fractions of the eluted peaks were taken and subsequently analysed to identify the component contributing to the activity in the AE22 extract. Identical conditions, using 0.1 M $NaNO_3$ (pH 8) as the mobile phase, were also employed in the chromatographic analysis of samples treated with the alcalase enzyme.

Viscosity

The viscosity of AE22, gum arabic and xanthan gum were compared using an Ostwald PST viscometer (Dannon-Fenske). All determinations were carried out at 21°C using a concentration of 0.2 mg/ml of each polymer dissolved in distilled water.

Statistical analysis

For the emulsification assays, a 3-way analysis of variance (ANOVA) was used to assess the significance of the emulsifying activities and K_d values compared to the control when averaged over all oil types and both pHs (Dunnett test). One-way ANOVA was used for each pH and oil type combination (Tukey's test) to determine if any significant difference to the activity and stability values produced by the three polymers. A p value of less than 0.05 was selected as the cutoff for significance.

Results

Isolation and strain characterization

During screening for the production of surface-active agents by various marine bacterial isolates, strain TG22 was selected for its ability to affect excellent emulsification against *n*-hexadecane. This strain was found to grow well in both ZM/10 and ZM/1 medium alone; however, maximum emulsification values (EI_{24}=60%) were obtained in the low nutrient concentration medium ZM/10 broth, supplemented with 1% glucose. TG22 was characterized as a Gram-negative heterotrophic bacterium that produces circular, convex white colonies that became off-white and sticky to ropy in aged cultures on solid medium. 16S rDNA sequencing identified the strain to be an alpha-proteobacterium of the genus *Antarctobacter*. GenBank accession number for TG22 is EF489005.

Growth and emulsifier production

Figure 1 shows the growth of TG22 in ZM/10 broth amended with glucose and production of its extracellular emulsifying activity. Exponential growth commenced immediately after inoculation, which was coupled with an overall gradual decrease in the pH of the medium from an initial value of 7.5 to 5.5 after 165 h. Emulsifier production in the cell-free culture broth was detected from 44 h, coinciding with the end of the exponential phase of growth, and reaching highest levels (EI_{24}=60%) well into the senescent phase of growth at 165 h. From an initial volume of 10 l ZM/10 broth amended with 1% glucose, the average

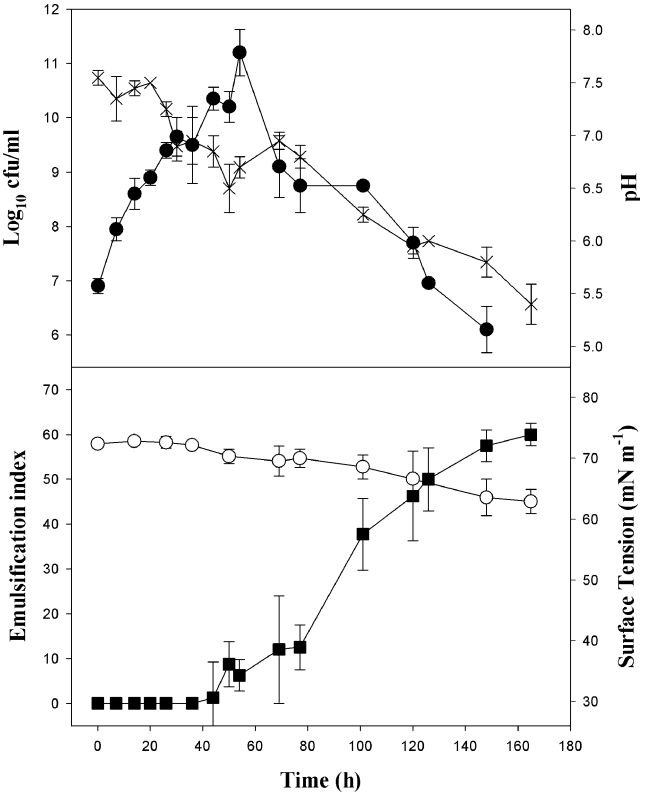

Fig. 1 Growth and emulsifier production by *Antarctobacter* sp. TG22 in ZM/10 liquid medium amended with 1% (w/v) glucose. Emulsification index and surface tension values were derived from cell-free culture broth after removal of the cells by centrifugation. *Solid circles*, Log_{10} cfu/ml; *x symbols*, pH; *empty circles*, surface tension; *solid squares*, emulsification index

dry-weight yield of the emulsifying extract AE22 recovered during the phase of maximal emulsifying activity (150–165 h) was 21.1 mg l^{-1}. During the course of the experiment, only a minor reduction to the surface tension of the culture broth was measured, from an initial value of 72.1 to 63.5 mN m^{-1} after 165 h. It is interesting to note that the emulsions formed remained stable for months without displaying any signs of phase separation or droplet coalescence (results not shown).

Chemical composition and molecular mass

The carbohydrate content of AE22 was 15.4±0.2% (Table 1) of the total weight of dried polymer recovered. Monosaccharide analysis showed that hexoses (rhamnose, fucose, galactose, glucose and mannose), amino sugars (galactosamine, glucosamine and muramic acid), and uronic acids (galacturonic and glucuronic acid) were present. Fucose (16.2±0.2), glucosamine (31.9±0.4) and glucuronic acid (20.3±0.7) were the most abundant, while all other monosaccharides were each present at less than 10% and together contributed about 31.5% to the total carbohydrate content.

Table 1 Monosaccharide profile of the extracellular emulsifier AE22

Component[a]	Mean mol% composition[c]
Rhamnose	4.9±0.1
Fucose	16.2±0.2
Galactose	0.5±0.0
Galactosamine	1.6±0.1
Glucose	3.0±0.2
Glucosamine	31.9±0.4
Mannose	9.6±0.0
Xylose	ND
Muramic acid	7.6±0.1
Galacturonic acid	4.3±0.2
Glucuronic acid	20.3±0.7
Total (%)[b]	15.4±0.2

ND Not detected

[a] *N*-acetylglucosamine and *N*-acetylgalactosamine are de-*N*-acetylated during the acid hydrolysis and are detected as glucosamine and galactosamine.

[b] Total percent values are expressed as the mean percentage of total dry weight of the polymer from triplicate determinations.

[c] Values are the mean of triplicate samples±standard deviation.

The total amino acid content of AE22 was 5.0±0.2% (Table 2) of the total weight of dried polymer. Amino acid analysis of hydrolysed samples identified the presence of three major amino acids—aspartic acid, glycine and alanine, which in total contributed 37.3% to the total amino acid content. The percentage contribution of polar amino acids to the total amino acid content was 60.8%, whereas that of hydrophobic non-polar amino acids was 39.2%. Lipid analysis did not reveal any fatty acids, indicating a large quantity of the polymer (up to 80%) was unaccounted for by our chemical analysis. This is not uncommon with some types of natural polymers that can be refractory to chemical analyses, as further discussed below.

Figure 2 displays the expansion of the proton NMR spectrum acquired from the AE22 extract showing strong peaks from the glycan component pre-dominating. Detailed analysis of the anomeric proton (H1) region (4.4–5.5 ppm) and the ring proton (H2–H5) region were not possible on these complex samples (some amino acid signals may also complicate this region), but several well-resolved features of the spectrum offer corroboration of the monosaccharide analysis. The peaks at 1.2–1.5 ppm are attributable to rhamnose and fucose H6 methyl groups. A resonance at 2.03 ppm is consistent with the presence of acetyl methyl groups of *N*-acetylglucosamine and *N*-acetylgalactosamine, thus indicating that at least some of the amino sugars are *N*-acetylated. However, the relatively low signal intensity, compared with that of the envelope of ring proton signals, seems inconsistent with the large amount of *N*-acetylglucosamine (31.9%) in Table 1, therefore suggesting that at least some of the amino sugars may be free and not associated with the polymer.

Chromatography of the AE22 extract on a PL–GFC column with 0.1 M $NaNO_3$ as mobile phase further corroborates the glycoprotein composition of the AE22 polymer. Four distinct refractive index (RI) peaks were eluted at retention times 8.7, 15.5, 16.7 and 18.3 min (Fig. 3), each displaying corresponding low-intensity UV peaks (absorption maximum at 280 nm), which is consistent with the low levels of total protein in Table 2. Analysis of fractions collected for each of these four peaks proved peak 1 to be the major component in the AE22 extract and to which all of the emulsifying, stabilizing and viscosifying activity were found to be associated with. The molecular mass of the active fraction (i.e. peak 1) was determined to be a molecule of more than 2,000 kDa. The remaining three peaks that eluted later were outside of the lower exclusion limit of the column (<2 kDa). These could be oligomeric degradation contaminants, such as free *N*-acetylglucosamine, because the high concentrations detected by monosaccharide analysis (Table 1) did not correspond to the weak signal for the acetyl methyl groups on the NMR spectrum (Fig. 2). Chromatography of AE22 using natural filtered seawater as a mobile phase showed the active fraction (i.e. peak 1 from Fig. 3) to be a heterogeneous mixture of at least two high-molecular-weight components, as shown by the irresolvable doublet peaks at retention time 8.7–9.2 min (Fig. 4). The

Table 2 Amino acid composition of the extracellular emulsifier AE22

Component	Mean mol% composition[a]
Asp	12.3±0.5
Glu	9.3±0.4
Ser	9.0±0.3
Gly	13.4±0.1
His	1.3±0.0
Thr	4.1±0.3
Arg	4.9±0.0
Ala	11.6±0.6
Pro	4.1±0.1
Tyr	1.3±0.1
Cys	1.9±0.2
Val	7.3±0.0
Met	ND
Ile	5.4±0.0
Leu	7.2±0.0
Lys	3.3±0.1
Phe	3.6±0.0
Total (%)[b]	5.0±0.2

ND Not detected

[a] Values are the mean of triplicate samples±standard deviation.

[b] Total percent values are expressed as the mean percentage of total dry weight of the polymer from triplicate determinations.

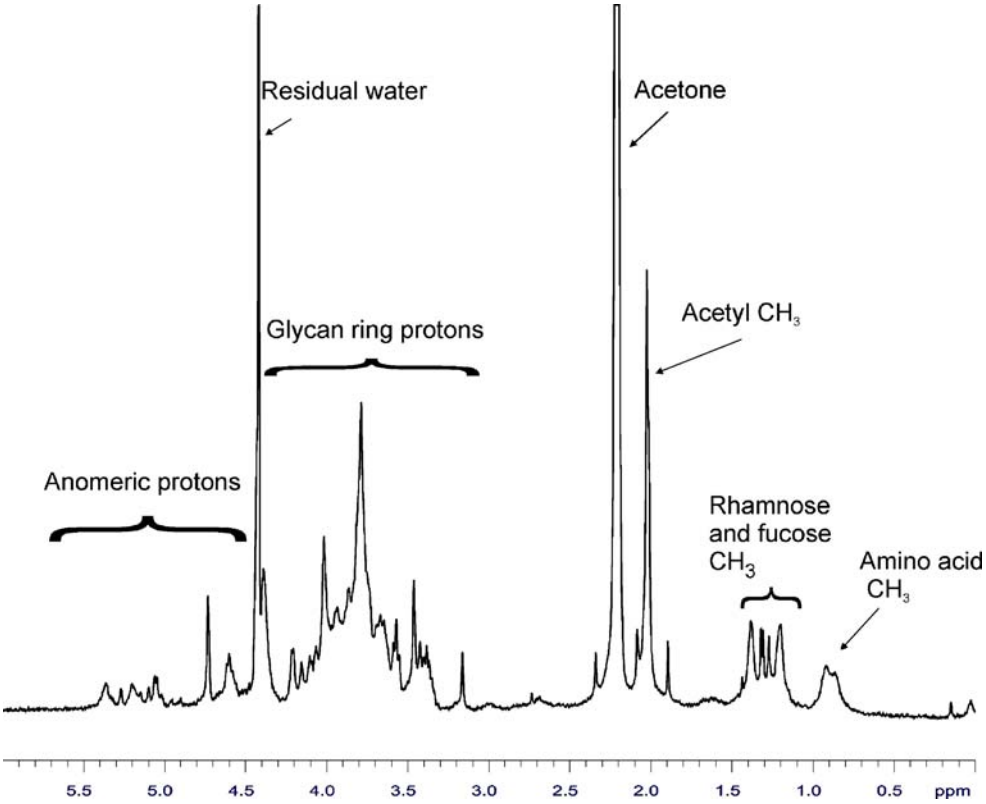

Fig. 2 Proton NMR spectrum acquired of the AE22 extract at 500 MHz, 60°C in D_2O. Assignments for some prominent signals in the spectrum are indicated; the remaining, unlabelled peaks arise from the protein component. Acetone is present as an internal standard (2.218 ppm)

relatively strong UV_{232} signals indicate carbon–carbon double bonding, possibly between the C4–C5 present in unsaturated uronic acids at the non-reducing terminal.

Treatment of the polymer with alcalase was successful in eliminating the minor protein fraction from the polymer, as evidenced by the disappearance of the corresponding UV peak with absorption maximum at 280 nm (Fig. 5). No observable shift to the RI signal of peak 1 occurred after deproteinization of the AE22 polymer by alcalase treatment.

Emulsification of different oils

Figure 6 shows the emulsifying activities and corresponding decay constants (K_d) of the different emulsifiers under

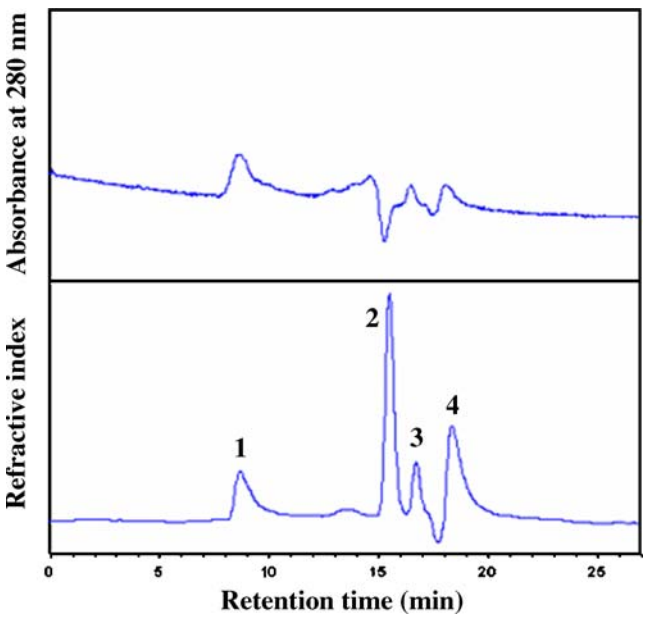

Fig. 3 Chromatography of the AE22 extract on a PL-GFC size-exclusion column with 0.1 M $NaNO_3$ (pH 8) as the mobile phase

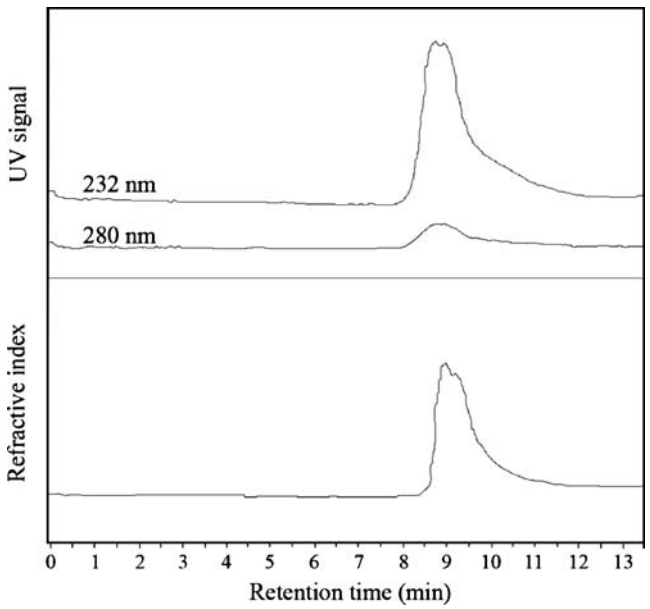

Fig. 4 Chromatography of the AE22 extract on a PL-GFC size-exclusion column with filtered seawater (pH 8) as the mobile phase

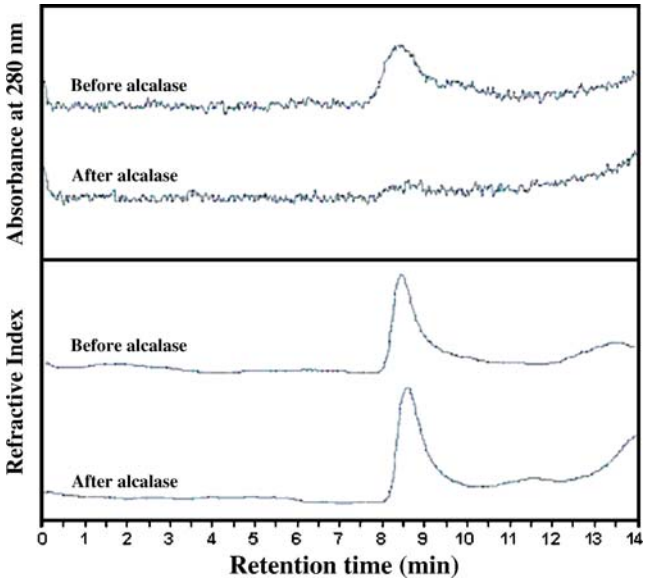

Fig. 5 Chromatography of the AE22 extract before and after treatment with alcalase. Samples were run on a PL-GFC size-exclusion column with 0.1 M $NaNO_3$ (pH 8) as the mobile phase

neutral pH (a and b) and acidic pH conditions (c and d) when tested against five different food oils. A higher K_d value denotes greater stability. A three-way factorial ANOVA showed that the main effect of polymer type was highly significant ($F_{3,80}=100.61$, $p<0.001$; data not shown). Averaging over all oil types and both pH values, only AE22 gave K_d values that were significantly different from the control (Dunnett test, $p=0.05$), whereas all three polymers (AE22, xanthan gum and gum arabic) gave emulsifying activities that were significantly different from the control.

A multiple one-way ANOVA analysis was used to identify significant differences ($p<0.05$) in emulsification activities and K_d values as contributed by the type of oil used and pH treatment. Under neutral pH conditions (Fig. 6a, b), AE22 and xanthan gum produced similar emulsifying activities against all the oils, except with vegetable oil where the activities of both AE22 and gum arabic were similar and significantly higher compared to xanthan gum. Gum arabic was the better emulsifier against olive and ground nut oil. Compared to the control, AE22 produced significantly more stabile emulsions against sunflower, vegetable and ground nut oil. Notably, its stability against ground nut oil was 8.4-, 5.9- and 5.4-fold higher compared to xanthan gum, gum arabic and no emulsifier, respectively.

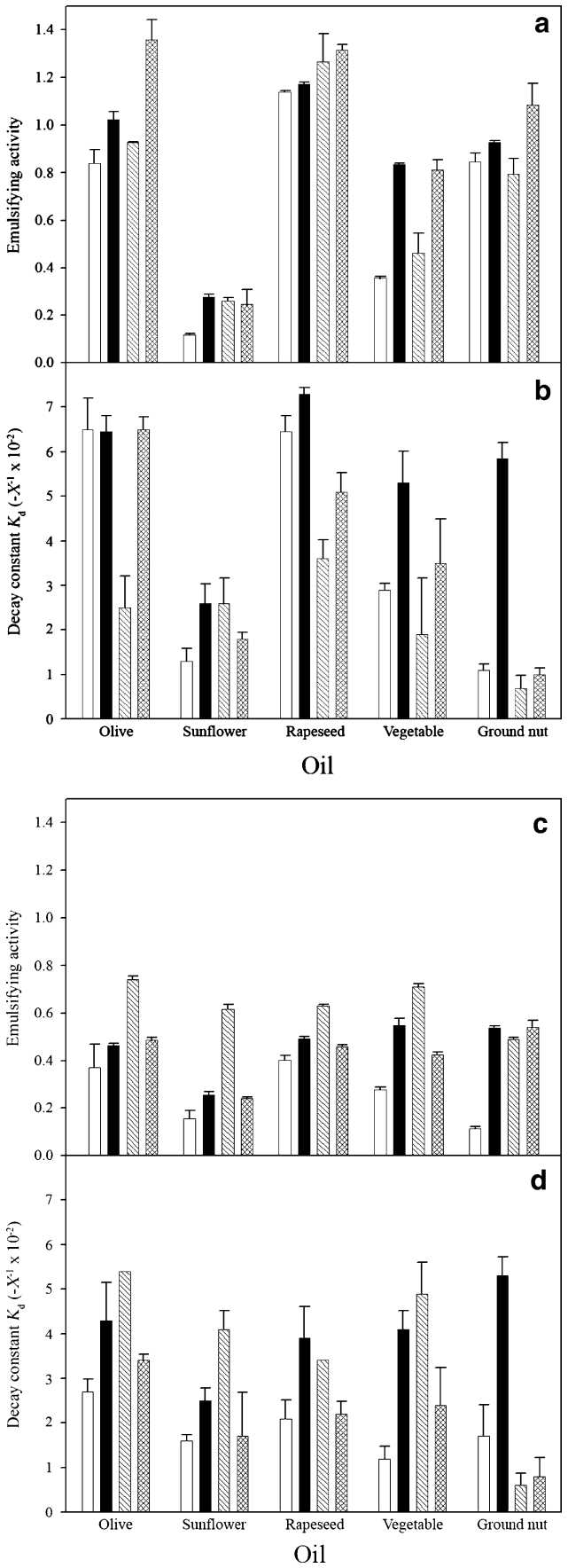

Fig. 6 Emulsifying activities and corresponding decay constants at pH 7.5 (**a** and **b**) and pH 3.5 (**c** and **d**) for O/W emulsions prepared using AE22 compared to the commercial emulsifiers. *Empty bar*, untreated control; *solid bar*, AE22; *diagonally shaded bar*, xanthan gum; *hatched (crisscross) bar*, gum arabic

Under acidic conditions (Fig. 6c,d), AE22 and gum arabic produced similar emulsifying activities with all the oils, except against vegetable oil where AE22's activity was higher. Overall, xanthan gum was the better emulsifier, producing significantly higher emulsifying activities than AE22 and gum arabic against all the oils except ground nut. The stabilizing effect of AE22 against olive oil was comparable to xanthan gum and gum arabic, whereas against sunflower oil, AE22 was comparable to gum arabic with xanthan gum as the better stabilizer. Both AE22 and xanthan gum showed similar stabilizing effects against rapeseed and vegetable oil. However, with ground nut oil in particular, AE22 produced emulsions that were significantly more stable—8.8-, 6.6- and 3.1-fold higher than those using xanthan gum, gum arabic and no emulsifier, respectively.

Surface tension and viscosity

Concentrations of AE22 (0.01 to 0.2% w/v) dissolved in water did not have any effect on the surface tension of water (72.1 mN/m at 21°C). However, the viscosity of water was significantly increased, and at concentrations of 0.02%, the reduced viscosity, η_{red}, was 2.19 $m^3\ kg^{-1}$, compared to 8.04 $m^3\ kg^{-1}$ for xanthan gum and 0.17 $m^3\ kg^{-1}$ for gum arabic.

Discussion

To our knowledge, this is the first report of an *Antarctobacter* species to produce an extracellular water-soluble polymer, for which its partial purification, chemical and functional characterization are presented. During growth in ZM/10 liquid medium amended with glucose, emulsifying activity was found coupled to the late exponential to senescent phases of growth. The emulsifying agent, AE22, could be readily extracted from the spent cell-free medium by solvent precipitation and the recovered material used to produce stable emulsions with hexadecane and various food oils. Relatively low concentrations (0.02%) of the AE22 polymer extract were sufficient to achieve stable emulsions, denoting a 'high yield value,' which is a useful property both in terms of process economics and potential biotechnological application (Sanderson 1990).

The chemical composition of the AE22 extract, as determined by monosaccharide, amino acid and GC analysis confirmed it to be a glycoprotein, composed of polysaccharide and protein but no lipid. This was confirmed by the proton NMR data, which displayed minor signals denoting the methyl groups of amino acids for the protein component and the presence of acetyl methyl groups of sugar amines and predominant glycan peaks for the polysaccharide. Further confirmation was provided by size-exclusion chromatography, which showed a minor protein component (maximum UV adsorption at 280 nm) coupled to a polysaccharide RI signal for the active component peak (>2,000 kDa). Chromatography using FSW as the mobile phase to simulate the natural conditions under which the polymer would exist and function in its native state (i.e. marine environment) proved the active component to be composed of at least two constituent high molecular weight (>2,000 kDa) biopolymers. Under conditions of salinity, dissociation of these constituents may be the result of disrupting H-bonding forces between these constituents. Although deproteinization of the polymer with alcalase was successful, a shift in the retention time of the active component was not observed, which could be explained by its elution above the exclusion limit of the HPLC column and beyond the columns effective resolution.

The three lower-molecular-weight species eluted during size-exclusion chromatography could be oligomeric degradation products of the large polymer molecule, possibly free amino sugars. This is supported by the relatively low intensity of the predominant signal at 2.03 ppm for *N*-acetylated amino sugars on the NMR spectrum, which, although accurate quantification is difficult, is inconsistent with the high concentration of these sugars that were found by monosaccharide analysis (e.g. 31.9% glucosamine).

It is interesting to note that almost 80% of the polymer was not accounted for by our chemical analysis. This feature is not uncommon and has also been described for other refractory exopolysaccharides, which in some instances has been attributed to the presence of uronic acids (Anton et al. 1988; Bejar et al. 1996) or glycosidic linkages of hexosamines (Biermann 1988). Such constituents can confer on polysaccharides a high resistance to degradation under acid hydrolysis conditions. As the producing strain TG22 belongs to a genus that is relatively poorly characterized and to our knowledge unknown for its exopolymer production, this polymer may contain unusual components that resist detection by the methods used in this study. Furthermore, the refractory nature of the polymer to heat-induced degradation is supported by preliminary results in our laboratory that have shown its viscosity and emulsion-stabilizing properties to be largely unaffected by high temperature treatments (unpublished results). Further work is presently underway to investigate this.

The amphipathic nature of the AE22 polymer in stabilizing oil-in-water emulsions and the observation that it was difficult to dissolve in distilled water suggest a partial hydrophobic composition. Certain chemical properties of polysaccharides, in particular some types of substituent groups, can give these large molecules amphipathic qualities, similarly to surface-active agents (Symes 1982). For example, the deoxy-sugars rhamnose and fucose, both

of which were identified in AE22, are recognized to express relatively apolar regions (Crow 1988; Graber et al. 1988). Similarly, a high uronic acid content could also contribute to emulsion-stabilizing properties, as has been reported with some galactomannans (Coia and Stauffer 1987; Garti and Reichman 1994) and xanthan gum (Ikegami et al. 1992). On the other hand, the proteinaceous material of some polysaccharides can also play a significant role in emulsion formation and stability, as exemplified by gum arabic which contains ~10% protein of the total gum (Dickinson et al. 1988; Nakamura 1986; Randall et al. 1989; Williams et al. 1990). Similarly, emulsan from *Acenitobacter calcoaceticus* (Kaplan et al. 1987) and other microbial-derived polysaccharides (Baird et al. 1983; Boyle and Reade 1983) have also been described to contain an active protein component. The non-polar amino acid fraction of AE22 (~39.2% of total protein) could therefore play a major role in its amphipathicity, particularly if these acids are orientated to the exterior of the polymer for maximal exposure and contact with the oil droplets within the continuous phase. Further work, however, will be needed to more accurately define AE22's amphipathic characteristics.

With a few exceptions, acidic conditions appeared to have an overall inhibitory effect on the emulsifying activity of all three emulsifiers (Fig. 6c,d). The reason for this may be due to protonation of carboxyl groups on the emulsifiers, as previously suggested by Rosenberg et al. (1979). Protonation of free fatty acids in the oils may also have contributed to this effect, and it is these acids that could explain the emulsifying activities observed in the untreated controls (i.e. no emulsifier added) (Erickson et al. 1980; Ma and Hanna 1999). Against most of the food oils tested, the emulsifying performance of AE22 was comparable to that of xanthan gum at neutral pH and to gum arabic at acidic pH. Most notable was AE22's ability to produce very stable emulsions under both neutral and acidic pH conditions, especially against ground nut oil. The results indicate that AE22 may be a better stabilizer than emulsifying agent. This quality, which is characteristic of natural hydrocolloid polymers, such as xanthan gum, is a useful characteristic for applications in healthcare and food oil formulations (Garti and Leser 1999). Although further work will be required to elucidate the mechanisms conferring AE22 with its high emulsion-stabilizing properties, its viscosity-enhancing properties could play a major role in this process.

While experiments are planned to more accurately define the role of the polysaccharide and protein component responsible for AE22's viscosifying and emulsion-stabilizing properties, at present, we can infer that this polymer is a high-molecular-weight glycoprotein with potential for further development as a hydrocolloid emulsifier. Its production by *Antarctobacter*, a genus that has received little attention and remains relatively poorly characterized, adds to our knowledge of microorganisms capable of producing exopolymers with potentially useful properties. AE22's polydisperse nature, as evidenced by its large molecular size and heterogeneous characteristics and its high percentage content of acidic residues, notably uronic acids, would suggest that it is likely to bind divalent ions effectively, as reported for other polymers with generally similar chemical properties (Arias et al. 2003; de Philipps and Vincenzini 1998; Moriello et al. 2003; Sutherland 1994). In this respect, the application of AE22 as a biosorbent material for the treatment of contaminated environments and industrial effluent is an interesting prospect worth investigating. Although further work is needed to better reveal the performance and functionality of this new glycoprotein emulsifier, particularly its production at an industrial scale, the results presented here show it has promise in biotechnological applications, particularly as an emulsion-stabilizing agent.

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